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Levesque, Mitchell P ; Cheng, Phil F ; Raaijmakers, Marieke I G ; Saltari, Annalisa ; Dummer, Reinhard

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Metastatic Melanoma Moves On: Translational science in the era of personalized medicine

Mitchell P. Levesque, Phil F. Cheng, Marieke I.G. Raaijmakers, Annalisa Saltari, Reinhard Dummer

University of Zurich, University of Zurich Hospital, Department of Dermatology, Gloriastrasse 31, CH-8091, Zurich, Switzerland

Abstract:
Progress in understanding and treating metastatic melanoma is the result of decades of basic and translational research as well as the development of better *in vitro* tools for modeling the disease. Here, we review the latest therapeutic options for metastatic melanoma and the known genetic and non-genetic mechanisms of resistance to these therapies, as well as the *in vitro* toolbox that has provided the greatest insights into melanoma progression. These include next generation sequencing technologies and more complex 2D and 3D cell culture models to functionally test the data generated by genomics approaches. The combination of hypothesis generating and hypothesis testing paradigms reviewed here will be the foundation for the next phase of metastatic melanoma therapies in the coming years.

Great discoveries often lag far behind technological innovations; real-world, transformative applications are even slower to materialize. It took 100-150 years to adapt the "reading stone" lenses of the 12th century into spectacles to correct hyperopia and myopia, and another two centuries to build telescopes out of lenses that allowed Galileo to support the Copernican model of a heliocentric solar system.

Although the pace of translational science has greatly accelerated since the middle-ages, the journey from theory, discovery, and application to measurable clinical benefit has been slow for most cancer therapies. This has been especially true for metastatic melanoma, which has only recently benefitted from decades of basic research. However, within the last 10 years, metastatic melanoma has gone from being almost untreatable to one of the most promising examples of evidence based, personalized medicine today. It was not until the publication of some landmark papers in 1996 and 2002 that the foundation was set for two of the most

disruptive technologies in the treatment of metastatic melanoma: targeted therapy and checkpoint inhibition [2-4]. These discoveries themselves were the results of decades of technological advances and basic research in next-generation sequencing technologies and immune biology, to name a few [5,6].

While the recent successes of single-agent and combination therapies have been encouraging, there is still a great deal of work to be done to better stratify patients for the appropriate treatment, to understand and circumvent therapeutic resistance, and to identify new targets for therapy. Below, we review the latest technological developments in these areas, the scientific results that will inform the next generation of therapies, and the challenges we still face in translating basic discoveries to treatments that will ultimately benefit patients. The selection of papers is focused on the technologies that have yielded the most clinically relevant results in recent years regarding metastatic melanoma progression: next-generation sequencing tools and *in vitro* functional assays.

Melanoma Treatment

The majority of malignant melanoma cases are detected at an early stage where surgical excision is curative. However, once melanoma has spread to a distant site, disease control becomes difficult. After decades of disappointing clinical studies in metastatic melanoma patients, the last 10 years have shown some exciting successes with clear clinical benefit. The current treatment paradigm for our metastatic melanoma patients follows the recently published treatment guidelines (Figure 1)[1].

Early studies in B-Rapidly Accelerated Fibrosarcoma (BRAF)-mutated patients investigated small molecules that blocked a range of kinases including RAF (sorafenib, RAF265), but more potent and selective inhibitors that targeted mutated RAF (particularly at amino acid 600) were more recently tested. These revolutionized the treatment of BRAF-mutant melanoma [7-11]. Phase III trials of both vemurafenib and dabrafenib demonstrated superiority over chemotherapy, as did the Mitogen Activated Protein Kinase Kinase (MEK1/2) inhibitor trametinib. However, within 5-7 months, disease progression was observed with single agent BRAF and MEK inhibitors [12-14]. In three randomized trials

(COMBI-d, COMBI-v, co-BRIM), combined targeting of BRAF/MEK (i.e., encorafenib and binimetinib, dabrafenib and trametinib, or vemurafenib and cobimetinib,) also showed improved response rates, progression free survival, and overall survival [15-20]. But resistance, (innate or primary, and acquired or secondary) is still a major challenge in the targeted-therapy paradigm.

To date, immunotherapy with high dose IL2 or checkpoint inhibitor antibodies against Cytotoxic T Lymphocyte Associated Protein (CTLA-4) and/or Programmed Cell Death Protein (PD1), and targeted therapies (BRAF and MEK inhibitors) in patients with a BRAF V600 mutation, have shown clinical benefit [1]. The use of BRAF inhibitors has significantly improved overall and progression free survival in BRAF V600 mutated patients [21]. Unfortunately, most patients develop resistance after some months.

Several theoretical models have been developed to understand how resistance may arise. These include a subclonal evolution model, phenotype switching, or cancer stem cells [6]. Like most models, each of these has some relevance and strong supportive data, but also major caveats. Understanding this complexity outside of the dogma associated with any particular model will facilitate a more accurate representation of how therapeutic resistance exists in a real-world clinical setting.

Genetic Mechanisms of therapeutic resistance (subclonal evolution)

There is some evidence that resistance may follow aspects of a Darwinistic evolution model in which selection and adaptation play vital roles. Small populations of resistant melanoma cells might already exist before treatment, either by genetic or epigenetic means, which are then selected during treatment due to the advantage they have over non-resistant cells [22]. Resistance could also develop during the course of treatment when the cells adapt to the new stressor. This too could happen via alterations in the genotype or epigenome resulting in phenotypic changes.

Immunohistochemical analysis of post-resistant samples from the phase II BRIM-2 vemurafenib trial revealed that nearly all resistant samples had a re-activation of the MAPK

pathway [23]. Since then, several genetic as well as non-genetic adaptive mechanisms for MAPK therapy have been described. A meticulous analysis of pre-treatment, on-treatment, and post-resistant biopsies has shown elevated pERK1/2 levels in progressive tumors and gain-of-function mutations in MEK and NRAS that reactivate MAPK signaling[24].

The first paper describing this reactivation demonstrated a de novo *MEK1* mutation (P124L) in a treatment-resistant metastasis from a BRAF-mutated patient who was successfully treated with a MEK inhibitor (selumetinib)[25]. This mutation conferred substantial resistance to MEK inhibitor therapy and only moderate resistance to a BRAF inhibitor. MEK mutations can also occasionally confer resistance to both BRAF and MEK inhibitors[26]. More commonly, concomitant NRAS mutations have been observed with persistent BRAF mutation{Nazarian, 2010 #2001;Shi, 2014 #5129;Van Allen, 2014 #5105;Raaijmakers, 2016, Co-existence of BRAF and NRAS driver mutations in the same melanoma cells results in heterogeneity of targeted therapy resistance}. This is especially interesting because of the paradoxical activation caused by BRAF inhibitors, which has been demonstrated for cutaneous epithelial malignancies[29,30]. It may be that the presence of an NRAS mutation at the point of progression to BRAFi-therapy may result in a detrimental effect because of this paradoxical activation.

In a large sequencing study that is described further in genomics section below, Shi et al analyzed the genomes of 100 tumor samples derived from 44 patients, which consisted of 29 pre-treatment samples and 71 resistant post-treatment samples [28]. All post-treatment samples still contained the $BRAF^{V600E/K}$ mutations, indicating that non-mutated subclones were not being selected during treatment. In line with the BRIM-2 analysis, the most common resistance mechanism they identified was MAPK reactivation (in 70% of the cases), either by an additional NRAS or KRAS mutation, mutant BRAF amplification or BRAF alternative splicing, or by CDKN2A loss. BRAF amplifications and truncated BRAF variants have also been reported elsewhere [31]. BRAF splicing variants result in a truncated BRAF protein, which lacks a RAS-binding domain, thereby conferring BRAFi-resistance through dimerization and pathway activation[32].

They also identified the PI3K-PTEN-AKT pathway as a second important resistance pathway (22% of their post-treatment samples contained mutations in PI3K-AKT regulatory genes).
By *in vitro* overexpression and knockdown studies, they could show that melanoma cells

overexpressing AKT1^{Q79K}, AKT1^{E17K}, AKT3^{E17K}, PIK3CA^{D350G}, PIK3CA^{E545G}, PIK3CA^{E545K} or PIK3R2^{N561D} were more resistant to BRAF inhibition compared to cells overexpressing the WT variant of AKT1, AKT3, PIK3CA and PIK3R2, respectively. In addition, PTEN knockdown in a PTEN WT cell line introduced vemurafenib resistance. When they analyzed 9 different resistant post-treatment samples from one patient, they identified at least 5 different resistance mechanisms, indicating that a single biopsy from one sample is not sufficient to determine follow up inhibitor treatment after BRAF inhibitor resistance.

Non-genetic mechanisms of therapeutic resistance (phenotype switching)

Disease progression occurs in most patients after successful tumor control with mono- or combination-therapy for months or even years. The response duration is generally quite broad (i.e., from weeks to years), and the causal mutations driving treatment failure are described above. In the context of the phenotype switching model, the transcriptional response to MAPK pathway inhibition can be considered to be proliferative cells activating mesenchymal (i.e., invasive or stem-like) expression modules that are associated with reduced glucose metabolism[33]. The melanoma cells may thereby buy time to activate alternative *signaling* pathways and enable subclones with additional activating mutations to flourish in the new microenvironment.

The activation of parallel *signaling* pathways such as the phosphoinoside-3-kinase (PI3K) pathway can also occur clinically, as well as MAPK-pathway reactivation [34-36]. This can occur, for instance, through the activation of the kinase COT [37]. The tumor stroma can also play an important role in bypass *signaling*. Stroma cells from resistant tumors can secrete growth and viability factors that protect melanoma cells from BRAF inhibition. These include the hepatocyte growth factor (HGF), which can facilitate tumor cell growth through the paracrine activation of the HGF receptor MET, which leads to PI3K pathway activation [38,39]. Other receptor-tyrosine kinases (RTKs) may enable proliferation in the presence of a BRAF inhibitor. While melanoma cells can express the epidermal growth factor receptor (EGFR) the levels are quite low compared to colon or squamous cell carcinomas. However, upon innate or acquired resistance, melanoma cells can upregulate the autocrine secretion of EGF and EGFR [40,41]. This suggests that, indeed, the tumor stroma is an activate participant

in the response to targeted therapies and may play a crucial role in the attenuation of therapeutic success.

Other avenues for intervention strategies may arise from a more complete understanding of the biochemical and metabolic events that are associated with MAPK reactivation, such as the maintenance of eIF4F complexes and the persistent ERK-independent phosphorylation of 4EBP1 or the enrichment of pro-apoptotic BCL-2-modifying factor (BMF)-dependent degradation of eIF4G [42]. Interestingly, interferon beta secretion was also observed to occur with tumor regression in the presence of BRAFi. In addition, PKCμ-phosphorylated ATF2 downregulates IFN² 1, which elicits therapeutic resistance (Lau et al. Oncogene in press).

Genetic mutations may confer permanent drug resistance. However, it has been observed that before an adaptive stage a transient and reversible drug resistance exists. Upon stress, such as inhibitor treatment, melanoma cells undergo an innate response which allows them to better tolerate drugs [43]. This response is characterized by de-differentiation (reduced expression of melanocytic lineage specific markers), increased expression of stemness and EMT markers and increased expression of genes involved in epigenetic remodeling. Of the latter group, the enzyme KDM5B is a marker for a slow cycling phenotype with increased drug tolerance [44,45]. This enzyme was upregulated in the stress response, as well as other markers, pointing to a downregulation of transcription and replication pathways, suggesting a slow cycling semi quiescent state. In this stationary phase cells change morphology to a flattened and enlarged phenotype and increased senescence associated SA-² -Gal activity, H3K9me3-positive heterochromatic foci and PML bodies [46]. Oher stressors, such as hypoxia or low glucose can induce a similar stress response [47]. Interestingly, when these stressed hypoxic or low glucose cells are exposed to various drugs, they are more resistant than their non-stressed equivalents.

In the previously mentioned study from Shi et al, the authors further investigated the genomes from 13 tumor samples derived from 1 patient [28]. Two samples were prior to treatment, 2 samples were taken during treatment, and 9 samples were disease-progressive tumors taken after treatment. The tumors taken during treatment expressed little to no Ki-67 staining in immunohistochemistry compared to pre-treatment samples, indicating a senescent like state with no proliferation. To the contrary, the post-treatment samples showed the highest level of Ki-67 staining; moreover, the 2 tumors from the post-treatment samples with the most

extensive genetic divergence displayed the highest proliferative activity, suggesting increased tumor fitness with decreasing C>T transitions, suggesting the presence of non-UV related mutagenic processes upon progression.

Early adaptive signaling upon BRAF inhibitor therapy can occur as soon as 24 hours after initiation of therapy [48]. Responses described include activation of the PI3K pathway [49], an altered oxidative metabolism [50] and upregulation of ERBB3 expression [51]. This rapid response, as well as the observation that melanoma primary tumors and metastases are very heterogeneous at multiple levels (i.e., genetic, epigenetic, transcriptional, proteomic, metabolomic, etc.) suggests that subclonal evolution or the stem cell model do not play much of a role in the acute phase of treatment response. This observation and some compelling publications over the last few years, have generated a widely accepted opinion in the melanoma community that these models alone fail to adequately account for heterogeneity and the early phases of resistance [52]. These subpopulations can contribute to tumor progression by exhibiting expression-driven, treatment-resistant slow-cycling behavior (such as JARID1B expressing cells) and tumor cells with features of epithelial to mesenchymal transition (EMT) or mesenchymal phenotypes and expression patterns. This plasticity may enable a dynamic response to external stressors such as therapies and may further generate a non-hierarchical organization of the tumor, which allows for a more rapid adaptation to the sudden reduction of MAPK signaling [53]. Interestingly, the limited cell death observed *in vitro* and *in vivo* during targeted therapy may be the result of proliferative tumor cells switching their epigenetic and transcriptional states to allow for a more slow-cycling mesenchymal phenotype that can better tolerate the therapeutic intervention. Resistance to BRAF inhibitors has also been associated with high, pre-treatment expression of anti-apoptotic BCL2 proteins (i.e., BCL2 and BCL2A1) [54,55]. This may explain the limited apoptosis that has been observed upon BRAFi treatment.

The role of MITF in resistance is conflicting, as roughly 50% of resistant tumors have an increased expression of MITF; whereas, the other 50% show a decreased expression, as an early response to BRAF inhibitors. Even within a patient, different relapsing tumors can have enhanced or absent MITF expression [56]. An interesting observation is that in (Vemurafenib) resistant cell lines, the presence of MITF indicated resistance to BRAF inhibition but retained responsiveness to MEK or ERK inhibition; whereas, absence of MITF was observed in cells that were also resistant to other MAPK pathway inhibitors [56]. The authors also noted that

cells without MITF expression can better tolerate higher drug doses. In addition, when they checked for intrinsic resistance in treatment naive melanoma cell lines, they found that if a BRAF or NRAS mutated melanoma cell line was intrinsically resistant to BRAF or MEK inhibitors, it had low MITF expression, which is probably due to the higher expression of RTKs such as AXL, EGFR and PDGFRb in MITF-low cells, as the inhibition of those receptors, especially AXL, sensitized the cell lines to MAPK inhibitors [56].

In cases of BRAF inhibitor resistant cells with elevated MITF expression, Nelfinavir (an HIV1-protease inhibitor), might be an interesting therapy [57]. Inhibition of BRAF or MEK reduces the expression of SKI, which leads to an upregulation of PAX3, and this in turn increases the expression of MITF [57]. In mice, combination treatment with Nelfinavir prevented the MAPK inhibitor-induced upregulation of PAX3 and MITF levels, thereby sensitizing the melanoma cells to MAPK pathway inhibition. However, whether elevated MITF expression is a direct cause of resistance has to be further evaluated.

Non-cell autonomous resistance

Interestingly, the classical theoretical models of melanoma progression tend to be cell-autonomous and do not leave much of a role for the interplay of external factors such as the tumor microenvironment. Darwin also postulated that species that learn to collaborate have a better chance to prevail. In a non-treated tumor, sensitive cells will generally have a growth advantage over resistant cells. However, upon treatment, resistant cells will have the upper hand, and it might even be so that sensitive cells support the resistant cells in this situation [58]. Upon treatment, sensitive cells secrete a therapy induced secretome (TIS), which was found to contain many mediators directly or indirectly activating the AKT pathway in resistant cells, who therefore became more proliferative upon treatment [58]. Treating mice with a combination of MAPK pathway and AKT pathway inhibitors could prevent this TIS induced accelerated expansion of resistant cells in tumors [58]. Resistant cells in turn can help sensitive cells by preventing dendritic cell maturation and activation. When treated with BRAF inhibitors, sensitive melanoma cells express danger signals that incite an immune response, the presence of resistant cells has a dampening effect on that response [59].

Collaboration can also take place with other cells in the tumor micro-environment. In a sophisticated *in vivo* mouse model where intravital imaging was combined with a biosensor construct that could monitor ERK/MAPK activity in live tissue, Hirata et al observed that melanoma cells respond to BRAF inhibition by reducing pERK; however, in tumor areas with high stromal density, cells rapidly recovered pERK expression to normal levels as soon as 1 day after the initial dose [60]. In the mice, these tumors were resistant, however when cultured as pure melanoma cultures *in vitro*, the cells were sensitive to BRAF inhibition, suggesting that the micro-environment aids melanoma cells to withstand inhibitor treatment. Indeed, when they co-cultured the melanoma cells with the tumor associated fibroblasts the melanoma cells conferred resistance to BRAF inhibition. It appeared that fibroblasts, upon treatment with BRAF inhibitors, paradoxically activate the MAPK pathway via PDGFRa upregulation which let them remodel the extracellular matrix in a denser collagen fibril matrix. This stiff matrix has changes in integrin organization and FAK signaling and directly provides a safe haven for melanoma cells. Adding FAK inhibitors to the BRAF inhibitor resensitized the melanoma cells to BRAF inhibition [60].

Lastly, next to tumor cell mutations and adaptations and micro-environmental factors, patient factors possibly also play an important role in resistance. How well the drug is absorbed in the intestine (e.g., receptors on intestinal cells, intestinal flora drug metabolism), how much of the drug is cleared in the liver (enzyme activity), how much of the drug is bound to albumin (starvation/feeding status), how well the drug can reach the tumor (vasculature, location of the tumor), all may have an influence on the drug concentration that effectively reaches the tumor cells. However, as every patient is unique, this is very complicated to investigate in an evidence-based medicine approach.

One of the factors that can be investigated and maybe compensated, however, is age. In a small cohort of 79 patients, those younger than 65 years had almost a double reduction in tumor burden upon MAPK inhibitor therapy than older patients, due to more resistance in the latter [61].
The authors found that sFRP2 levels are increased in the serum of patients older than 55 years compared to younger patients, probably due to an increased secretion by aged senescent fibroblasts. This increased sFRP2 expression caused a reduction in b-catenin, MITF and APE1 expression. A loss of APE1 leads to more cellular damage by ROS. Indeed, melanoma cells exposed to aged fibroblasts or aged fibroblast conditioned medium *in vitro* have greater

DNA damage. This damage could be reversed by inhibiting ROS activity. Decreases in b-catenin and MITF and increases in ROS have been linked to BRAF inhibitor resistance. The authors could show that *in vitro* and *in vivo* melanoma cells treated with conditioned medium from young fibroblasts or implanted in young mice were more sensitive to BRAF inhibition than those treated with the conditioned medium from old fibroblasts or implanted in old mice, respectively.

Melanoma Genomics

Undoubtedly, one of the major transformative technologies driving translational research today is next-generation sequencing (NGS). Although genetics has been known to contribute to melanoma progression for decades, the first pivotal study identifying BRAF as the most important driver gene revealed that melanoma is a genetic disease and highlighted the importance of the MAPK pathway [3]. It was the discovery of BRAF V600 that was mutated in 50% of the melanoma samples with V600E comprising 80% of the mutations. Another observation from this study was that NRAS mutations, mutated in 15% of the melanoma samples, were mutually exclusive to BRAF mutations [3]. The scope of this study was just focused on the MAPK pathway (i.e., RAS-RAF-MEK-ERK), but revealed crucial information about the genetics of melanoma. Many subsequent NGS studies used whole exome (WES) or whole genome sequencing (WGS) on melanoma tumors, melanoma short term cultures or melanoma cell lines. In this section we will focus on the large cohort studies for a comprehensive review of genomic analyses in melanoma [62].

The first genomic characterization of melanoma was a whole genome sequencing study performed on the melanoma cell line COLO-829 [63]. An ultra-violet radiation (UVR) signature, defined by C > T substitutions [64], was detected in this cell line. This finding supported the notion that UVR is an environmental risk factor for melanoma. Two of the largest (WES) studies looking at 121 melanoma tumors and 147 melanoma tumors confirmed the UVR signature for sun-exposed melanomas and uncovered that non-sun-exposed melanomas exhibited a lower mutation rate and different mutation signature, thus highlighting the differences in melanoma subtypes, such as acral, mucosal, and uveal melanomas [65,66]. These two studies together detected known melanoma oncogenes such as BRAF and NRAS and in addition uncovered new oncogenes like RAC1, PPP6C, and STK19 and new tumor suppressors like ARID2, DCC, TACC1, SNX31, NF1, ZNF560, FAM58A, and ME1. Copy

number analysis revealed losses in known tumor suppressors like PTEN and CDKN2A and gains in known melanoma oncogenes like MITF, CCDN1, CDK4 and TERT. These two studies provided a great insight into the genomic landscape of melanoma and also highlighted the fact that driver mutations in BRAF and NRAS do not likely occur from UVR. This suggests that the initial oncogenic transformation is UV independent. Exome sequencing of $BRAF^{V600E}$ mice treated with UVR revealed a typical UVR mutation signature across the exome and inactivating mutations in *Trp53 [67]*,thus demonstrating the direct link of UVR to melanomagenesis.

WGS of 25 metastatic melanomas revealed frequent PREX2 mutations and TERT promoter mutations [68,69]. WGS also confirmed the UVR signature and as well revealed many structural rearrangements in the genome that after known oncogenes like ETV1. TERT promoter mutations were also seen in an independent study looking at families with a history of melanoma and were also validated in sporadic melanoma samples [70]. WES has also been used for phylogenetic analysis of metastatic progression of melanoma [71,72]. Each study had 8 patients where the primary tumor and multiple metastases were subjected to WES. Both studies found that metastasis is not a simple linear progression of mutation accumulation, but exhibits a quite complex evolution from the primary tumor. The metastases can be founded from multiple subgroups of the primary lesion. These findings have great implications for therapy as inter-tumoral and intra-tumoral heterogeneity can play a large role in response to treatment.

The next largest omics study came from The Cancer Genome Atlas melanoma working group [73]. In this study, 67 primary tumors and 266 metastatic tumors were analyzed by WES, RNAseq, copy number, and reverse phase protein array (RPPA). Two new molecular subtypes were defined in this study. Tumors that were both BRAF and NRAS wildtype often had a mutation in NF1 (45%). Since no high frequency hotspot mutations occurred in NF1, it was assumed that NF1 was a tumor suppressor. NF1 is known as a negative regulator of RAS, thus loss of function mutations would lead to activation of the MAPK pathway. Tumors that were triple wild-type for BRAF, NRAS and NF1 were typically less likely to have a UVR signature and were more likely to have copy-number alterations and complex structural rearrangements. Interestingly, there were no significant mutations associated with metastasis suggesting progression to metastasis may be patient specific.

The next two WES studies analyzed 501 and 213 melanomas and confirmed the NF1 molecular subtype. In addition, RASA2 mutations were discovered to significantly co-occur with NF1 mutations [74,75]. It was observed that the NF1-mutated melanomas had a higher mutation rate than BRAF or NRAS mutated melanomas suggesting that NF1 alone is not sufficient to drive melanomagenesis and additional mutations are needed such as mutations in RASopathy genes. RASopathies are developmental disorders caused by mutations in genes in the RAS/MAPK pathway. For instance, germline mutations in NF1 cause neurofibromatosis. Many melanoma mutations share known RASopathy gene mutations, reviewed in [76], suggesting that patients with a RASopathy could have a higher chance of developing melanoma.

One large WES study interrogated the mutation progression of melanocytic nevi to melanoma [77]. This study sequenced 37 FFPE melanomas with histologically distinct precursors. The authors sequenced by WES,150 distinct areas. Benign nevi generally had one mutation, which was usually BRAF V600E. Intermediate lesions had at least two pathogenic mutations and had a higher mutation rate than benign lesions. BRAF V600E mutations occurred in intermittent sun-damaged skin and in younger patients, whereas BRAF V600K or K601E and NRAS mutations occurred in chronic sun-damaged skin and older patients. TERT promoter mutations were found in a significant portion of the intermediate lesions. Once a melanoma becomes invasive, inactivation of CDKN2A occurs. TP53 and PTEN mutations were observed to occur in late stages of invasion. This study elegantly dissected the stepwise genetic evolution from melanocytic nevi to invasive melanoma.

Melanoma transcriptomics

One of the first studies to apply RNAseq to melanoma samples discovered 11 novel gene fusions and 12 novel chimeric transcripts and in addition, validated 29 somatic mutations in 10 melanoma samples [78]. The study did not detect any of the gene fusions in an additional 90 samples suggesting gene fusions are private events.

The TCGA has one of the largest collections of RNAseq data with 470 melanoma samples with supporting clinical data. From the TCGA melanoma landmark study, RNAseq analysis uncovered 3 signatures of gene expression across these melanoma tumors: a group with high immune gene expression, a group with high keratin gene expression, and a group with low

MITF gene expression. Survival analysis of these three groups revealed the keratin group to have the lowest survival rate and the immune group to have the highest survival rate, suggesting biological significance to these gene signatures. BRAF hotspot mutations were more likely to be found in the MITF-low group (66%), and the MITF-low group generally contained samples with a mutation in BRAF or NRAS or NF1 compared to the keratin and immune group (3% versus 21% and 14%, respectively).

Before NGS, microarrays were a popular tool to dissect the molecular characteristics of melanoma. Microarray analysis of 218 melanoma cell cultures across 6 different studies revealed two gene expression patterns, a "proliferative" signature and an "invasive" signature [79]. The "proliferative" signature was defined by high expression of typical melanocytic genes like MITF, TYR, LEF1, and SOX10 and the "invasive" signature was defined by high expression of WNT5A, SOX9, TGFB, and TCF4. This signature was found to be independent of primary or metastatic lesions and of mutated BRAF V600E. These two signatures had phenotypic consequences *in vitro*, with the "Proliferative" signature melanoma cells having a fast doubling time and limited invasive capacity, whereas "invasive" signature melanoma cells had a slow doubling time and high invasive capacity, and the melanomas were able to switch back and forth between the two phenotypes [80]. This has been termed phenotype switching. A recent study demonstrated that the TCGA RNAseq dataset also contained these two signatures [81]. No significant mutations were associated with either phenotype suggesting these two cell states are driven by transcriptional reprogramming and not by genetic events. We have recently shown that DNA methylation of the SOX9 promoter is at least one of the mechanisms that regulates this phenotype by the mutually exclusive developmental transcription factors SOX10 and SOX9 that could control aspects of each phenotypic state (Figure 2) [82,83].

Single cell RNAseq has also dissected the heterogeneity of melanoma [84]. In this study, 4645 cells were isolated from 19 melanoma tumors and subjected to single cell RNAseq. One interesting finding from the study was the MITF high signature and an AXL high signature which resembled the proliferative and invasive signatures described before. At the bulk tumor level, the tumor could be classified as MITF high or AXL high, but at the single cell level the spectrum of mutual exclusive expression of MITF and AXL could be found. The tumor also contained cells with high cycling capacity and low cycling capacity. Along with the tumor cells, stromal cells and lymphocytes were also sequenced. Cancer associated fibroblasts

(CAFs) had a significant association with the AXL high signature and an anti-correlative association with the MITF high signature. CAFs and melanoma cells could both express an AXL signature suggesting tumor-stromal interactions where CAFs could influence the transcription profile of melanoma cells. CAFs expressing C3 also had a significant association with $CD8^+$ T cells. Briefly, the lymphocyte population could be distinguished by their specific identities and subsets by known gene markers and even identifying exhausted T-cells. Exhausted T-cells were linked to T cell expansion, whereas non-exhausted T-cells were not expanded. Overall, this study has provided great insight into the transcriptomic heterogeneity of melanoma and the interaction of melanoma cells with the environment of stromal and lymphocytic cells.

Discovery of resistance mechanisms to therapy by genomics and transcriptomics

NGS has also been a great boon for the study of drug resistance mechanisms in melanoma. One of the first studies using gene expression profiling discovered that upregulation of PDGFRB leads to resistance to BRAF V600E inhibition [27]. In that same study, mutations and upregulation of NRAS also conferred resistance to BRAF V600E inhibition. Targeted sequencing of 138 cancer genes uncovered a MEK1 mutation (C121S) to confer resistance to BRAF V600E inhibition [85]. WES of 20 patients uncovered $BRAF^{V600E}$ copy number gain [86]. WES of FFPE material from 45 patients revealed MEK2 mutations and some MEK1 mutations that confer resistance to Vemurafenib [87]. MITF amplification was also detected in this study to confer resistance. Multiple resistance mechanisms were also seen to evolve in parallel within one tumor. One patient had two distinct NRAS mutations that were detected mutually exclusively on reads that spanned the same locus. The authors suggested that the two mutations occurred *in trans* in the same resistant tumor or represent separate subclonal resistant populations in the tumor.

Earlier, we described the WES treatment-resistance study of Shi et al [28], which detected 22% of the resistant tumors had a perturbation in the PI3K-PTEN-AKT pathway, which included mutations in AKT1/3, PIK3CA, PIK3CG, PIK3R2, PTEN and PHLPP1. The authors observed that 20% of the resistant tumors could have more than one resistance mechanism, suggesting tumor heterogeneity and/or collaborative mechanisms. Phylogenetic analysis of multiple resistant tumors from the same patient revealed branched evolution rather than linear evolution suggesting resistant mechanisms can evolve in parallel in different tumors.

RNAseq analysis of the same patient cohort revealed that transcriptional processes were highly consistent in the resistant tumors, which is in contrast to genetic events that were more heterogeneous [88]. cMET upregulation and LEF1 downregulation occurred in the majority of resistant tumor cells. These two genes were observed to have a strong anti-correlation with DNA methylation, suggesting an epigenetic mechanism to BRAF inhibitor resistance. In addition, upregulation of M2 macrophage markers could be seen in a subset of the tumors which was also associated with reduced expression of T cell markers. The authors suggest that the involvement of the immune system is also an important component to BRAF inhibitor resistance. It is interesting to note the importance of LEF1. It is a gene part of the proliferative signature in the phenotype switching model. We have observed that cells with a proliferative phenotype are generally sensitive to BRAF inhibition and upon resistance the cells adopt an invasive signature, thus supporting the notion that down-regulation of LEF1 leads to resistance [33]. In the single cell RNAseq study by Tirosh, the authors also observed that cells adopt an invasive signature, AXL high MITF low, when resistance occurs [84]. These studies highlight the role that phenotype switching has in resistance to BRAF inhibitors (see Kemper et al. for a comprehensive review [89]). Again, based on our initial findings published in cancer research 2008 [80] these two profiles have been predicted and coined proliferative (MITF high) and invasive (MITF low AXL positive) phenotypes [90]. Resistant tumors are characterized by a strong expression of gene sets that we have called invasive gene sets representing mesenchymal transition, cell adhesion, extracellular matrix remodeling, angiogenesis and stemness [80].

Immunotherapy with anti PD-1 antibodies is a preferred first-line therapy for metastatic melanoma [91]. However, not all patients respond, which is also true for anti-CTLA4 therapy. Therefore, intensive research is focusing on the properties of responding versus non-responding tumors. WES has also been used to correlate clinical responses to anti CTLA-4 blockade [92]. In this study 110 patients were subjected to WES and 40 of those with RNAseq, they observed that high mutational load, neoantigen load, and expression of cytolytic markers were beneficial for response. WES and RNAseq was performed on a set of 38 and 28 melanoma patients treated with anti PD-1 to elucidate resistance mechanisms and biomarkers [93]. Mutational load did not have a significant association to response to anti PD-1 therapy and neoantigen load was not significant for response either. BRCA2 mutations occurred in 30% of the responders to anti PD-1. However, the frequency of BRCA2 mutations

may differ between anti-PD-1 responders and nonresponders. Previous studies have shown that tumor mutational and neo-antigen load were correlated with response to anti-PD1 immunotherapy in non-small-cell lung cancer and colon cancer {Rizvi, 2015, Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer}{Le, 2015, PD-1 Blockade in Tumors with Mismatch-Repair Deficiency}. Interestingly, resistant tumors expressed a gene signature reminiscent of the invasive phenotype. AXL, ROR2, WNT5A, LOXL2, and TWIST2 were significantly upregulated in the non-responding tumors. The authors also noted that this gene signature is also seen in BRAF inhibitor resistant tumors suggesting an innate biological response to anti-tumor therapy. In another study looking at PD-1 resistance mechanisms, JAK1 and JAK2 mutations were found in two patients that developed new lesions under anti PD-1 treatment. B2M mutations were found in one other patient. JAK1 and JAK2 mutations cause loss of interferon gamma growth induced arrest [94].

During the evolution of a malignancy there is an intensive interaction with immune cells described as immunoediting[95]. This process can be subdivided into three phases: the elimination phase, the equilibrium and the escape phase. During the elimination phase immune cells such as T-lymphocytes recognize neoantigens on tumor cells and are able to kill most of them. However, some tumor cells survive and manage to grow, but this growth is controlled by immune response mechanisms [5]. This is called the equilibrium phase. If the tumor cells gain additional mechanisms to suppress immune functions the tumor cells can escape. We speculate that one of these escape mechanisms is the up-regulation of immune suppression factors such as PDL-1 on the surface of tumor cells.

For the immunoediting process, expression of HLA surface molecule is essential, and is controlled by interferons. Interestingly the genomic analysis of acquired resistant tumors during anti-PD1 therapy showed in two of the four patients resistance-associated alterations of the interferon signal (loss of function mutations in Janus kinase 1 or Janus kinase 2). In addition, a mutation of the Beta-2-microglobulin, which is an essential competent of HLA class 1, was found in another patient [96].

In summary, NGS has provided a wealth of information about the genomic and transcriptomic landscape of melanoma biology and resistance. These studies have shown the genetic and

transcriptional events important for melanoma progression as well as resistance to BRAF inhibitors and immunotherapy. Of note is the phenotype switching model, which seems to have an important role in the fundamental behavior of melanoma and in one of the resistance mechanisms to targeted therapy and immunotherapy.

In vitro tools of melanoma metastasis

While the strength of NGS studies is an unprecedented profiling of large scale molecular events, finding causal features from high-dimensional data remains a great challenge. Thus, *in vitro* tools such as diverse cell line biobanks that better represent actual tumor diversity [97], more complex but scalable assays that serve as relevant tumor proxies, and *in vivo* models that are translatable to human biology are essential to test the hypotheses generated by NGS projects. Here we will summarize the latest *in vitro* developments that will facilitate the functionalization of genes identified in WES or RNA-seq experiments.

As melanoma is the most aggressive form of skin cancer with a high predisposition to invade and metastasize [98], it is of fundamental importance to select the most appropriate *in vitro* model to study disease progression ex vivo [99,100]. For many years, melanomas have been studied by using cell lines that grow under adherent conditions to form two-dimensional (2D) monolayers. However, 2D cultures do not fully reproduce the tumor three-dimensional (3D) organization, the cell-cell and cell-matrix interactions and the patchy distribution of oxygen and nutrients. For this reason, 2D monolayers do not represent an adequate system to study the tumor biology and the acquired resistance to therapeutic treatments. In fact, the drug response observed in monolayers often fails to reflect the *in vivo* situation [101,102]. To date, 3D models have been used in melanoma research for preclinical studies as a compromise between standard 2-dimensional cultures and xenografts of human tumors, taking advantage of their reduced costs and time compared to animal models [103].

Three-dimensional melanoma spheroids

Among 3D tools, melanoma spheroid models mirror the architecture of the tumor and recreate the oxygen/nutrient gradients observed *in vivo* [102]. Comparative gene expression profile studies revealed that genes associated with proliferation, differentiation, resistance to therapy and migration are differentially expressed in cells maintained as 3D spheroids in comparison

with 2D cultures suggesting the preferential expression of specific constellations of genes in well-defined structures [104-106]. Moreover, the localization of the cells expressing specific markers can be affected by the model used. For example, ERK is mainly found in the growing periphery of spheres, mirroring the sub-compartmental expression of melanoma lesions. On the contrary, it is homogeneously expressed in 2D cultures [107].
Similarly, the choice of *in vitro* models is also relevant in the context of targeted therapy. One of the best examples comes from BRAF inhibitors (BRFAi) such as vemurafenib and dabrafenib. The inhibition of growth and invasive abilities of 3D melanoma spheroids with these drugs recapitulates the results of phase II and III clinical trials.

Melanoma spheroids can be classified into two different groups: 1) multicellular tumor spheroids (MCTSs), and 2) tumorospheres, Although these models share the same 3D structure, they are used for different applications according to their intrinsic features[108]. In contrast to tumorospheres that select a subpopulation of melanoma cells with stemness properties, the multicellular spheroid model (MCTSs) preserves the cell heterogeneity of the tumor [100]. MCTSs can be generated with different protocols, all of them based on the anchorage-independent growth, such as the "liquid overlay method", the "hanging drop method", the encapsulation in alginate-based membranes or with rotating systems [108]. In the liquid overlay method, cells are seeded on plates previously coated with a thin layer of inert material (agarose or polyHEMA) to prevent cell adhesion and promote aggregation. Alternatively, cells can be placed in commercial ultra-low attachment plates. The "hanging drop technique" consists in depositing small drops of cell suspension on a lid to induce the cells to accumulate and give rise to spheroids [109]. MCTSs can also be obtained after encapsulation in microparticles of well-defined and reproducible structure and size, which are compatible with high-throughput screening studies. Finally, MCTSs formation can be induced by using rotating systems (shakers and spinner flasks). Implanting multicellular spheroids into collagen I coated surfaces allow the study of melanoma migration and invasion. This assay is frequently used to study the metastatic potential of melanoma as it mirrors the intercellular interactions with the tumor microenvironment [110-112]. To further investigate the role of stromal cells in the tumour behaviour, MCTSs can be generated from a co-culture of melanoma and different cell types (fibroblasts, endothelial or immune cells).

The second 3D tool to study melanoma biology is the tumorosphere. Unlike MCTSs, their main goal is not to fully replicate the *in vivo* tumour, but rather to isolate melanoma cancer

stem cells (CSCs). However, whether this model really enriches CSCs is still a matter of debate [113]. The formation of tumorospheres spontaneously occurs when cells are plated at a low density in the presence of serum-free conditioned media ("stem cell medium") supplemented with basic fibroblast and epidermal growth factors (bFGF and EGF). The resulting spherical aggregates derive from the clonal expansion of one single cell [114].

In the recent years, two new protocols have been developed to generate tumour spheroids such as the tissue-derived tumor spheres (TDTSs) and the organotypic multicellular spheroids (OMSs). While TDTSs are generated by partial dissociation of cancer tissues, OMSs are derived from tumor fragments maintained in culture without any dissociation step [115].To date, these models have been successfully generated only for glioblastoma, lung, bladder and colorectal cancer, but none of them has been established for melanoma [115].

Analysis of melanoma spheroids with imaging software and mathematical models

Given the great complexity of the 3D structures and the presence of cells on different focal planes, the analysis of melanospheres is not easy and obvious. For this reason, a huge number of software tools have been developed with the aim to analyze growth, invasion and drug response. The majority of these protocols allow for the interpretation of the behavior of spheres through the calculation of a set of parameters (diameter, total area, area of invasion, factor shape, percentage of fragmentation, number of invading cells, distance reached in collagen etc.), thereby defining a complete picture of the model [116]. To this purpose, spheroids are followed up and pictures are taken at different time points to assess the changes in the tumor morphology and size. Pictures are then converted into binary images and analyzed, manually or through an automated system, with an imaging software (ImageJ, CellProfiler, SpheroidSizer, AnaSp, Image-Pro Analyzer etc.). In addition, some mathematical models can mirror the distribution and penetration of the drug within spheroids, contributing to highlighting the kinetics and dynamics of the treatment [117]. Although it will be difficult to achieve a general protocol of analysis, its standardization could simplify the complexity of the available mathematical models and encourage industries to consider this as a standard step for the evaluation of drug efficacy.

Three-dimensional melanoma skin equivalents

One of the critical aspect of melanoma progression is the invasion of the dermal compartment after crossing the basement membrane. The 3D melanoma skin reconstructs represent a better tool to study the mechanisms underlying this early stage of the tumour and to evaluate the toxicity of new therapeutic approaches on the healthy cells of the skin.

Melanoma skin reconstructs are artificial skin consisting of a dermal equivalent, composed of fibroblasts embedded in collagen, and an epidermal compartment, composed of keratinocytes and melanoma cells [102]. This model is representative of the tumour microenvironment and architecture. In addition, melanoma reconstructs accurately recapitulate the different stages of tumour development. It has been demonstrated that melanoma cells are located in the skin equivalents in a different position according to the progression stage from which these cells are derived [118]. In detail, cells from radial growth phase tumours are confined to the epidermis, while those derived from vertical growth phase melanomas are located at the dermal-epidermal junction. This model has been used in the past to evaluate the efficacy of BRAF inhibitors, revealing a decreased proliferation and the induction of apoptosis in melanoma cells [119]. Despite the great utility of skin reconstructs in testing the efficacy and toxicity of new compounds, this model is a time-consuming procedure that requires constant monitoring and more than 15 days to obtain the skin. Moreover, it can be employed only to evaluate the early stages of the tumour, when melanoma cells are still in the dermis. On the other hand, it is the *in vitro* tool that closest resembles the tumour architecture, and will undoubtedly play an important role in hypothesis testing from NGS studies prior to the application of even more difficult and costly *in vivo* models.

Outlook: New therapeutic strategies

If melanoma progression is thought of in a Darwinistic evolutionary model, this opens the possibility of adapted therapeutic dosage schemes with already available drugs [120]. This might suggest that the goal should be to find an equilibrium between the resistant and sensitive tumor cells to control tumor proliferation. Without therapy, sensitive cells have an advantage over resistant cells, whereas the opposite is true with therapy. By applying drug holiday schemes, in theory, the two populations could then be better controlled [121]. But

therapeutic or microenvironmental phenotype-switching of cells into slow-cycling, treatment resistant subclones can add even more complexity to an already complicated phenomenon [47,52]. Thus, future treatments will have to account for the large variety of resistance patterns that exist, which include innate, adaptive, fixed (i.e. genetic), and non-cell autonomous resistance. Targeting multiple pathways may not only make sense from a resistance perspective, but also for controlling both phenotypic states of melanoma cells.

Clinically, tumor regression after termination of MAPK pathway inhibitor treatment is sometimes seen, but overall disease regression or even clinical remission has not been described. Due to tumor heterogeneity and different resistance mechanisms (i.e., both genetic and adaptive) present in an individual patient, not all tumors within one patient will react in the same way upon treatment; hence it will be very difficult to adjust therapy schemes accordingly. We think future efforts will need to focus on better sampling of tumor material, as one tumor biopsy may not represent a complete tumor or a complete patient. The collection of liquid biopsies (circulating tumor cells) might be an approach that gives a better picture of the heterogeneity within one patient [122]. Likely, combination treatments targeting different pathways or hitting the same pathway twice, or combining therapies with different working mechanisms, will be necessary to induce overall disease regression, after which new therapeutic dosage schemes and strategies can be applied.

There have been unexpectedly great successes in the last decade of translating basic research results into treatment strategies with measureable clinical benefits. But we still need better tools to analyze molecular phenomenon with greater breadth and precision, and also to build better theoretical and *in vitro* models that will more accurately recapitulate the complexity inherent in actual patient tumors. The speed of discovery keeps accelerating, and our efforts to bring that new knowledge to the clinics should keep pace.

Figure Legends:

Figure 1: Current treatment guidelines for metastatic Melanoma [1].

Figure 2: Current model of melanoma heterogeneity (i.e., phenotype switching), whereby SOX9 and SOX 10 represent mutually exclusive repressors of the alternative fate and switching of phenotypic states is driven by microenvironmental factors and epigenetic programming [6,123,83].

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